"Remedial effect of *Phyllanthus acidus* against Bleomycin provoked Pneumopathy"

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Summary

The model most often used to study the pathogenesis of pulmonary fibrosis in the Bleomycin model (BLM) induced lung fibrosis, several treatments has been efficacious but not in the clinic. To examine the effect of *Phyllanthus acidus* against Bleomycin induced pulmonary fibrosis in rats the methanol extract prepared by soxhlet extraction method and used for the animal study. The animals received single intra-tracheal injection of Bleomycin (2.5 U/kg). The treatment animals received (500 mg/kg) of *Phyllanthus acidus* extract by oral gavages for a week prior after BLM treatment and Hesperidin (200 mg/kg) used as standard drug. Animal killed by lethal dose of sodium pentobarbital. The lung was removed the biochemical estimation carried out in lung homogenate. The total cell count, differential cell count, total protein, Alp, TNF-α level estimated in lung BALF. The DNA damage, histopathological changes assessed in lung tissue. The depletion of SOD, CAT, GPx, level in Bleomycin treated animal compare to control. The herbal drug regulate the normal level of enzymatic antioxidant due to free radical scavenging activity, it also control the lipid peroxidation and decrease MDA level. The plant extract regulate the normal level of total cell counts and differential cell counts. The PAE normalize the level of protein, Alp, TNF-α in the BALF compared to standard drug. The plant extracts scavenging ROS reduce DNA damage and tissue damage.

Key Words: *phyllanthus acidus,* pneumopathy, Bleomycin model.

Introduction

Idiopathic pulmonary fibrosis is a chronic inflammatory intestinal lung disease of potential fatal prognosis and poor response to available medical therapy. It has been hypothesized that activated inflammatory cells which accumulate in the dower airways release increased amount of reactive oxygen species [ROS] which, combined with a deficiency in glutathione the major component of the lung injury and fibrosis, the antioxidant has shown beneficial effects in disease in which ROS appear involved. The model most often used to study the pathogenesis of pulmonary fibrosis in the Bleomycin model (BLM)-induced lung fibrosis, several treatments has been efficacious but not in the clinic. To examine the effect of phyllanthus acidus against Bleomycin-induced pulmonary fibrosis in rats

Experimental Method

Plant materials:-

Phyllanthus acidus leaves used for this study were obtained from (host plant) in Deviyakurichi, Salem district, Tamilnadu, India. The plant leaves were identified by Botanical Survey India, Coimbatore, and The voucher samples are kept in the BSI herbarium for reference **(Bsi/sc/5/23/08-09/tech-613).**

Preparation of Plant Extract[4]:-

The fresh leaves of *phyllanthus acidus* collected from the host plant were first washed free of sand and debris. The plant leaf carefully shade dived at room temperature. A quantity of the ground sample was soxhlet extracted using methanol. The extract was evaporated to dryness at 45-50°C.

Phytochemical Studies:-

The chemical classes of constituent in freshly prepared extract were detected using standard phytochemical reagent and procedures as described by herbone. In general, test for the presence, absence of phytochemical compound using the above methods involve the addition of an appropriate chemical agent to the crude material in a test tube. The mixture is then shaken vigorously of gently as the case may be, the presence or absence of alkaloids, flavonids, triterpenoids, flavonolds, tannins, saponins etc, was observed.

Animal model[6]:-

In this experiments thirty healthy male Wistar strains rats, three months of age, weighing 120 – 150 g were selected for acclimation for a period of two weeks in laboratory animal house and maintained under standard conditions of temperature 27 ± 2°C, relative humidity of 60 ± 5% and 12:12 hour light: dark cycle prior to experimentation. The animals were fed with standard pellet diet and water. The experimental animals were divided into five groups each contains six animals as per the drug treatment plan. First group served as control and the rest served as experimental group. All animal experiments were conducted as per the instructions of Institutional Animal Ethics Committee **(NCP/IAEC/PG/02/2009)**.

Experimental Groups and Treatment Regimen: -

The animals were randomly divided into five groups with six animals each under identical conditions and were given dose, Group-I. Control animal received normal saline.Group-II. Animals received the (2.5 U/kg) Bleomycin in a single Intra-tracheal inj. Group-III. Animals received *phyllanthus acidus* extract (500 mg/kg) by oral gavages for a week period.Group-IV. Animals received standard drug Hesperidin (200 mg/kg) by oral gavages for of week period after Bleomycin administration. Group-IV. Animals received *phyllanthus acidus* extract (500 mg/kg) by oral gavages for a week period after Bleomycin administration.The group I, II, III, animal was killed after 7 days, group IV, V were killed after treatment conclude by lethal injection of sodium pentobarbital (100 mg/kg) followed by exsanguinations abdominal aorta. The lung was removed and a piece fixed in 10% formalin for histology. Lung homogenate was prepared for the bio chemical study.

Biochemical estimation[4,5]:

The Superoxide dismutase activity estimated by Kakkar et al., (1984) method using NADH-DMS-NBT, Catalase (CAT) by the method Sinha (1972). Glutathione peroxidase (GPX) activity was assayed by the method Rotruck et al., (1973) Malondialdehyde (MDA) measured by Nichons and Somualson (1968).

Broncho Alveolar Lavage[7]:-

The lung BALF was obtained by washing the lung four times with 4 ml of aliquots saline through a tracheal cannula. Cell suspensions concentrated by low speed centrifugation, and cell pellet resuspended in saline. Total cell counts were made in a haemocytometer, differential cell counts was determined by cytospin preparation by counting 100 cells stained with May

Grunwald Giemsa. Total protein content in BALF supernatants was measured by Lowry method[8]. ALP measured by laboratory method[9]. TNF α was measured by enzyme-linked immunoassay according to the manufacture protocol (lab system).

Analysis of DNA fragmentation in lung tissue[10]:-

The animal's lungs were minced with a razer blade and suspended in a mixture of 100 ml HCl (P^H 8.0) 40 mm Na-EDTA, 10 ml NaCl, 1% Sodium decodyl sulphate (SDS). The samples were incubating at 50°C overnight and then subjected to phenol chloroform (1:1) extraction. After ethanol precipitation the DNA were dried. The pellet was re-suspended in 30 ml of 10 mm fix HCl (P^H 7.5) and 1 ml EDTA, The micrograms of DNA were electrophrosed on a 7% Agarose gel stained with Acridine orange G photographed by under UV Trans illuminator.

Histological Evaluation:-

After death the right lung tissue were fixed by inflation buffered with a 10% formalin solution for 24 hour and after being embedded in paraffin. A midsagittial section of each lung was cut at 3μm thickness and stained with hematoxylin and eosin. The pathological grade made of inflammation and fibrosis in a whole area of a midsagittial section was evaluated with oil immersion (100X) magnification by light microscopy. A pathological grade was analyzed by MD Pathologists.

Result and Discussion

Biochemical estimation

The rat lung homogenates was used for the estimation of enzymatic antioxidant Superoxide dismutase, Catalase, Glutathione peroxidase level and oxidant product Malondialdehyde level. In the present study depletion activity of SOD level in Bleomycin treated rats compare to control animal. The therapeutic treatment with *Phyllanthus acidus* herbal drug significantly improved the SOD level compared with standard drug Hesperidin. The inhibition of Catalase activity during Bleomycin-induced toxicity was due to the increased generation of reactive free radicals. It can create an oxidative, an oxidative stress in the cell. The administration of *PAE* increased the Catalase activity in lung tissue and protected from free radical-induced oxidative stress. GPx activity significantly increased in Bleomycin treated rats compared to saline treated group, treatment with *phyllanthus acidus* were significantly decrease in the level GPx to normal level**.** The levels of MDA in Bleomycin treated group significantly increased compared to control group animals. The Administration of herbal drugs *PAE* at the therapeutic treatment showed the

maximum reduction compared to standard drug Hesperidin. The result was presented in the **Table- 1.**

Broncho Alveolar Lavage

The total protein, alkaline phosphate, tumor necrosis factor alfa level estimated, also the total cell counts and differential cell count carried out in the rat lung broncho alveolar lavage fluid. Protein in Broncho alveolar lavage fluid was significantly increased in Bleomycin in treated rats compared to control group. The BALF total Protein from the *PAE* treated rats showed a tendency to exhibit reduced protein level. The *PAE* regulates the protein near to normal level compared with the standard drug Hesperidin. The control group shows the normal level of the ALP. Bleomycin treated rats ALP level significantly increased in the BALF. The *PAE* reduces the ALP compared to the standard drug Hesperidin. The *PAE* alone group also shows the normal level of ALP. In the present study Bleomycin treated group increased the tumor necrosis factor level in BALF compared with standard drug. The inhibitory effect TNF production may certainly contribute to the beneficial effect the antioxidants. *Phyllanthus acidus* having the antioxidant that may reduce in the TNF level in the treatment group animals. This result showed in the **Table-2.**

The total cell count in Broncho alveolar lavage fluid were significantly increased in Bleomycin treated rats compared to rats not exposed Bleomycin. *PAE* alone was without effect on total cell counts. The Administration *PAE* in therapeutic effect, it levels total cell counts to normal counts compared with the standard drug Hesperidin**.** The differential cell counts showed that Neutrophils were markedly increased in Bleomycin treatment compared to control group Esoinophils counts levels also slightly increased in the Bleomycin treated group Broncho alveolar lavage fluid inflammatory cells. The *PAE* treatment group differential cells counts levels normal compared to that standard drug Hesperidin group. The PAE alone group not shows any change in differential cell count. The results were showed in the **Table -3**

Analysis of DNA fragmentation in lung tissue:-

In the present study Bleomycin treated group DNA smear shows the fragments compare to control group, it indicates Bleomycin radicals causes the DNA damage. The results showed in the **Figure1 and 2**. The administration herbal drug *Phyllanthus acidus* on therapeutic treatment reduces the DNA fragment compare to standard drug Hesperidin. The results were showed in the **Figure 4 and 5**. The *PAE* alone group DNA smear shows no fragments, it indicates PAE didn't cause DNA damage. The results showed in the **Figure 3**

Histological Evaluation:-

Histological examination of lung tissue from control group displayed a normal structure and no pathological changes under the light microcopy. The lung tissue from Bleomycin treated animals revealed significant tissue damage compared with lung section taken from saline treated animals, these were characterized by extensive of inflammatory cells including Neutrophiles and lymphocytes in alveoli and intersitium damaged alveolar epithelial cell. The results were showed in the **Figure 7**. The administration of herbal drug *phyllanthus acidus* on treatment significantly prevent the lung inflammation compared with standard drug Hesperidin group. The results were showed in the **Figure 9 & 10**. The *PAE* alone group displayed a normal structure of lung tissue. The result were showed in the **Figure 8**

Table-1 Effect of *Phyllanthus Acidus* Treatment on Lung Cells Oxidant Products and Anti - Oxidants

S.No	Experimental groups	Mean values of enzymatic antioxidant			Mean values of oxidant product
		SOD	**CAT**	**GPx**	**(MDA)**
1	Group I	0.83U/mg protein	0.62nMol/mg protein	60.88µMol/mg protein	0.210nMol/mg protein
2	Group II	0.092U/mg protein	0.30nMol/mg protein	51.50µMol/mg protein	0.870nMol/mg protein
3	Group III	0.75U/mg protein	0.60nMol/mg protein	62.10µMol/mg protein	0.195nMol/mg protein
4	Group IV	0.81U/mg protein	0.65nMol/mg protein	61.25µMol/mg protein	0.182nMol/mg protein
5	Group V	0.72U/mg protein	0.58nMol/mg protein	55.75µMol/mg protein	.172nMol/mg protein

Table-2 Effect of *Phyllanthus Acidus* on BALF Total Protein, ALP, and TNF-Alfa

S.No	Experimental groups	Total protein	ALP	TNF-α
1	Group I	6.8 µg/ml	60mU/ml	70ng/ml
2	Group II	10.2µ g/ml	188mU/ml	325ng/ml
3	Group III	7.2µg/ml	66mU/ml	58ng/ml
4	Group IV	6.5µ g/ml	71mU/ml	64ng/ml
5	Group V	7.6 µg/ml	77mU/ml	79ng/ml

Table –3 Effects of *phyllanthus acidus* on Broncho alveolar lavage fluid cell profile

S.No	Experimental groups	Total cells	Neutrophills	Lymphocytes	Esoinophills
1	Group I	9800cells/cumm	54%	43%	03%
2	Group II	13,450cells/cumm	71%	20%	09%
3	Group III	8500cells/cumm	58%	40%	02%
4	Group IV	8800cells/cumm	52%	44%	04%
5	Group V	8100cells/cumm	57%	38%	05%

Effects *phyllanthus acidus* on DNA Fragmentation in Lung Tissue

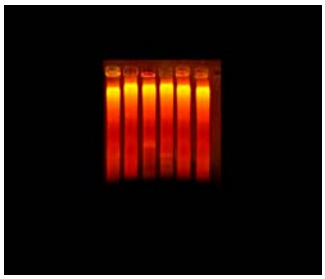
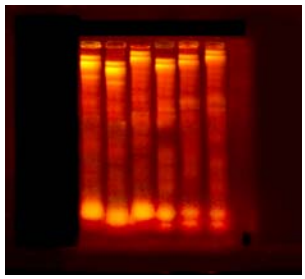


Figure 1



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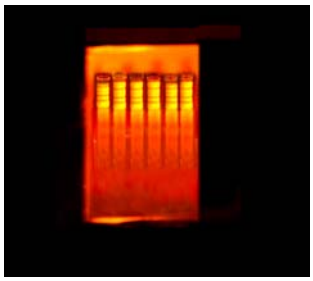

Figure 3

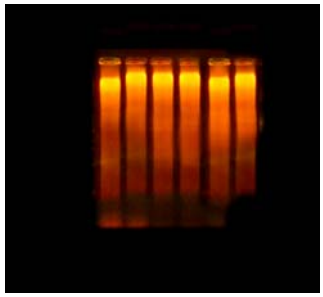

Figure 4

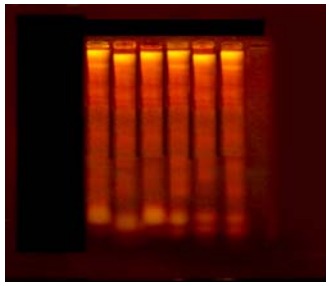

Figure 5

Effects of phyllanthus acidus on Alveolar inflammation and lung fibrogensis in rats with pulmonary fibrosis

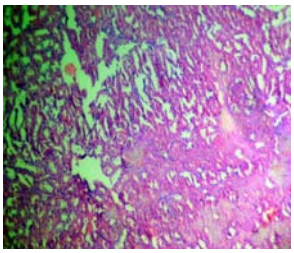

Figure 6

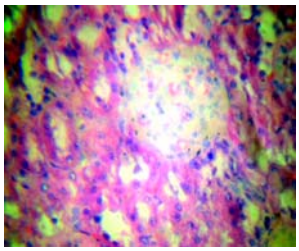

Figure 7

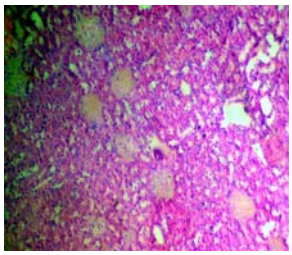

Figure 8

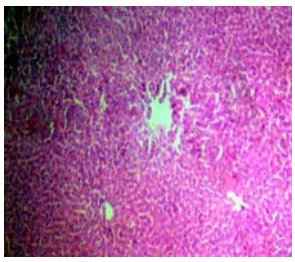

Figure 9

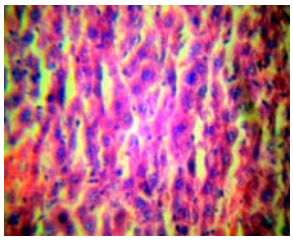

Figure 10

Conclusions

In this present study clearly suggest that the administration of *Phyllanthus acidus* cure the Bleomycin-induced pulmonary fibrosis. (1) By free radical scavenging activity. (ii) By interfering with the influx of inflammatory cells (iii) by suppressing the activation of alveolar macrophages (IV) by prevention collagen accumulation in lung.

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